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14. ABSTRACT

The objective of this proposal is to determine the effectiveness of tumor stromal targeting using radiolabeled antibodies to deliver cytotoxic payloads to breast cancer stromal fibroblasts. The central hypothesis to be tested is that targeting the breast cancer stroma will result in enhanced tumor cytotoxicity compared to targeting the breast cancer cells themselves. Fibroblast activation protein (FAP) is a cell surface glycoprotein selectively expressed by tumor stromal fibroblasts in breast tumors, but not significantly expressed by breast cancer cells, normal fibroblasts, or other normal tissues. We have identified an appropriate animal model that allows for evaluation of both stromal and epithelial targeting using BT-474 xenografts. In multiple biodistribution experiments using I^{125} radiolabeled antibodies targeting either HER2 or FAP, epithelial targeting was accomplished. However stromal targeting of FAP remains suboptimal, most likely due to the relatively low copy number of FAP in the tumor stroma compared to tumor antigens. Although targeting the tumor stroma was challenging utilizing the antibody reagents tested in this proposal, additional antibody reagents are under development including higher affinity antibodies to overcome the challenges encountered. The biodistribution experiments conducted can inform future therapeutic studies to investigate a radioimmunotherapeutic strategy for treatment of breast cancers.

15. SUBJECT TERMS

stromal fibroblast, radioimmunotherapy, Fibroblast Activation Protein

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INTRODUCTION

Clinical effectiveness of radioimmunotherapy (RIT) in lymphomas has led to the FDA approval of the radiolabeled antibodies Ibritumomab Tiuxetan and Tositumomab. Some of the major impediments for successful use of RIT in patients with breast cancer include: 1) insufficient selectivity of the tumor antigen leading to host toxicities, and 2) poor tumor penetration of large antibody molecules due to the impaired vascular access and high interstitial pressure of tumors. These obstacles may be overcome if an exquisitely tumor-selective antigen in the tumor stroma is targeted with antibody fragments that more easily penetrate the tumor mass. Fibroblast activation protein (FAP) may be such a target. FAP is a fibroblast cell surface glycoprotein that is selectively expressed by tumor stromal fibroblasts in breast tumors, but is not significantly expressed by breast cancer cells, normal fibroblasts, or other normal tissues [1]. We have shown that FAP potentiates tumor growth *in vivo* [2].

The objective of this proposal is to determine the effectiveness of tumor stromal targeting using radiolabeled antibodies that deliver cytotoxic payloads to breast cancer stromal fibroblasts expressing FAP. **The *central hypothesis* to be tested is that targeting breast cancer stroma will result in enhanced tumor cytotoxicity compared to targeting the breast cancer cells themselves.** Targeting the tumor stroma may be advantageous due to the close physical proximity of the tumor stromal cells to the tumor capillaries, allowing easier accessibility of circulating antibodies in the blood to their targets. Since stromal cells have fewer genetic alterations compared to transformed malignant cells, reactive stromal cells may express target antigens in a more uniform and stable fashion compared to malignant cells.

BODY

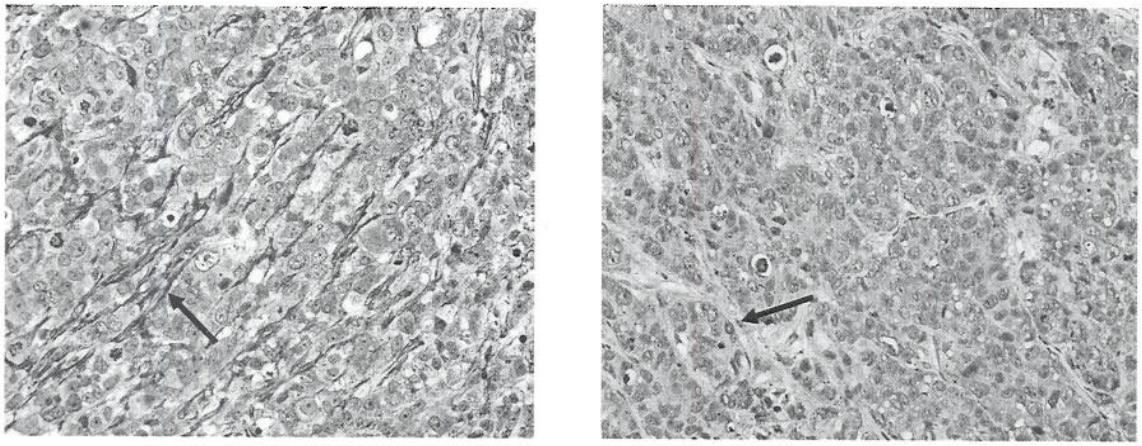
The primary objective of this proposal is to characterize the effectiveness of radioimmunotherapy employing antibodies that target stromal FAP in a breast cancer xenograft model. This proposal aims to test the hypothesis that radiolabeled diabodies targeting FAP in the breast cancer stroma will have enhanced therapeutic effectiveness compared to radiolabeled antibodies targeting HER2 on the breast cancer cells.

Identification of Breast Xenografts with FAP Induction

A number of breast cancer xenografts were stained for stromal fibroblast induction of FAP. These breast cancer cell lines included BT474, MCF7, MD231, and MD361. BT474 had the most robust induction of stromal FAP as seen in **Figure 1** below. Paraffin embedded tumor specimens were deparaffinized to distilled water using xylene and alcohol. Microwave antigen retrieval was performed in 10 mM Citrate Buffer ph 6.0 for 10 minutes. After peroxidase and protein blocking, the primary anti-FAP rabbit antibody was applied in a dilution of 1:1200 overnight, and the secondary goat anti-rabbit incubated for 30 minutes. Avidin-biotin complex streptavidin-horseradish peroxidase was applied prior to the addition of chromogen-DAB substrate. Specimens were counterstained with Hematoxylin and dehydrated to xylene. The arrow in **Figure 1, Left Panel** demonstrates intense stromal staining of FAP in BT474 xenografts, as contrasted by the lack of stromal

staining (arrow) in the MD361 xenograft (**Figure 1, Right Panel**). Thus, BT-474 breast cancer xenografts induce robust stromal FAP expression, and were utilized for radioimmunotherapeutic evaluations.

Figure 1. Immunohistochemical staining of FAP in breast cancer xenografts

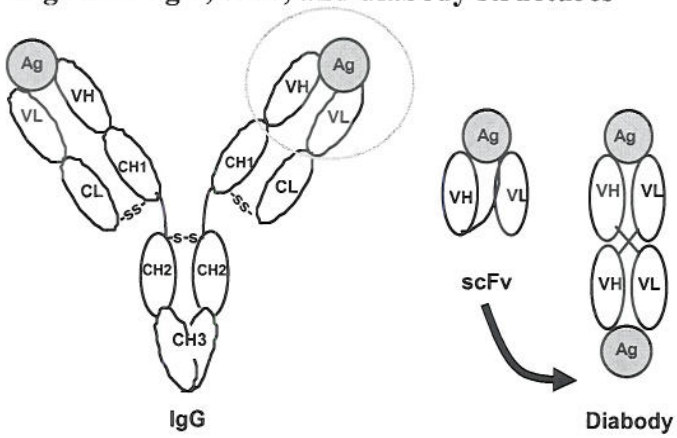


FAP (+) BT474 Xenografts **FAP (-) MDA-MB-361 Xenografts**

Anti-FAP antibodies

Single-chain antibodies (scFv) were identified using phage display techniques by panning a naïve human phage display library containing 10^{10} unique phage [3] against recombinant murine FAP. The scFv antibody E8 was identified as an antibody targeting murine FAP and converted into a diabody format for its favorable pharmacokinetic properties and prolonged *in vivo* half-life. As shown in **Figure 2,** the variable heavy (VH) and light (VL) domains of an IgG molecule are joined by a 15 amino acid spacer to form a single chain Fv (scFv) molecule capable of binding a single antigen (Ag). Shortening the spacer to 5 amino acids prevents association between the light and heavy chains of a single scFv molecule. Their high affinity for each other favors the formation of noncovalent dimers (diabodies).

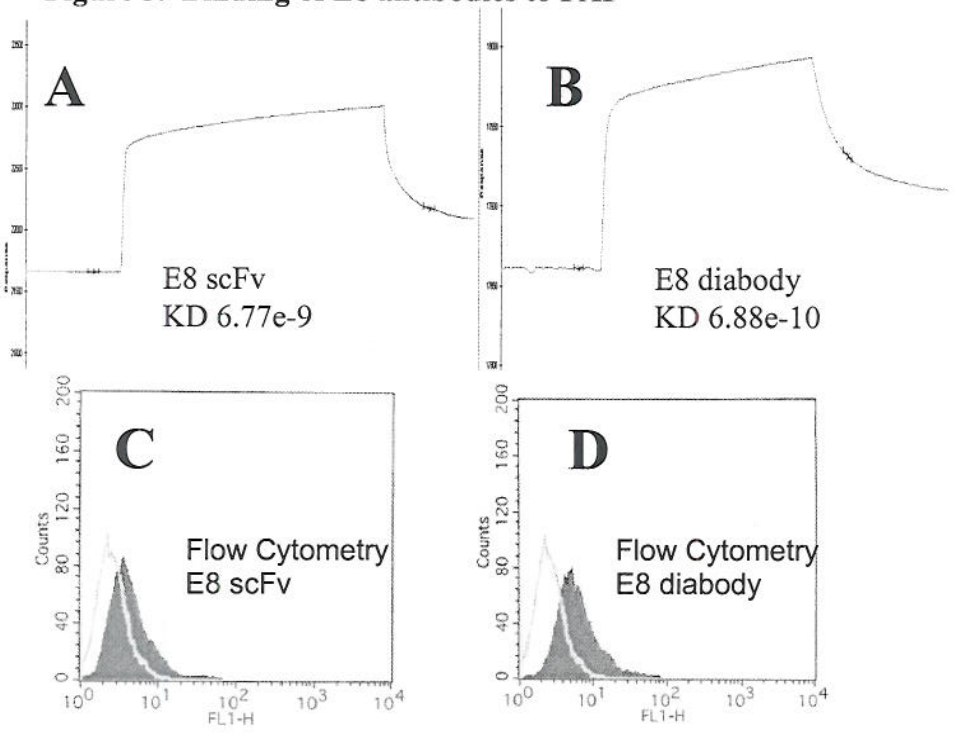
Figure 2. IgG, scFv, and diabody structures



These antibodies were characterized by BIAcore and FACS analysis [4]. Surface plasmon resonance (SPR) was performed using BIAcore technology. The SPR response reflects the change in the mass concentration as molecules bind to or dissociate from a ligand, immobilized on a sensor chip. ScFv association/dissociation rates and affinities of the E8 scFvs and diabodies for FAP was determined by passing serially diluted samples of the scFv over a FAP-ECD immobilized CM5 chip. The sensograms in **Figure 3** show the interaction of the scFv E8 (**Figure 3, Panel A**) and E8 diabody (**Figure 3, Panel B**) with the immobilized FAP-ECD, demonstrating a K_d of 6.77 x 10^{-9} M and 6.88 x 10^{-10}M for E8 scFv and diabody, respectively.

Flow cytometry was performed using HEK293 cells stably transfected with FAP. HEK293-FAP cells were incubated with the scFv primary antibody on ice, followed by a secondary mouse anti-His antibody directed against the His epitope tag of the scFv, followed by FITC-conjugated goat anti-mouse tertiary antibody, and fixed with 1% paraformaldehyde in PBS. HEK293-FAP cells incubated only with the secondary and tertiary antibodies were used as a negative control. The samples were analyzed on a BD FACScan Instrument as shown in **Figure 3**. The scFv E8 (**Figure 3, Panel C**) and E8 diabody (**Figure 3, Panel D**) demonstrates FAP specific binding as seen by the right shift of the closed curve of the E8 FAP antibodies compared to the open negative control curve of non-specific His antibody. The E8 antibodies did not bind to the negative control cells of HEK293 transfected with vector only (mock) controls (data not shown).

Figure 3. Binding of E8 antibodies to FAP

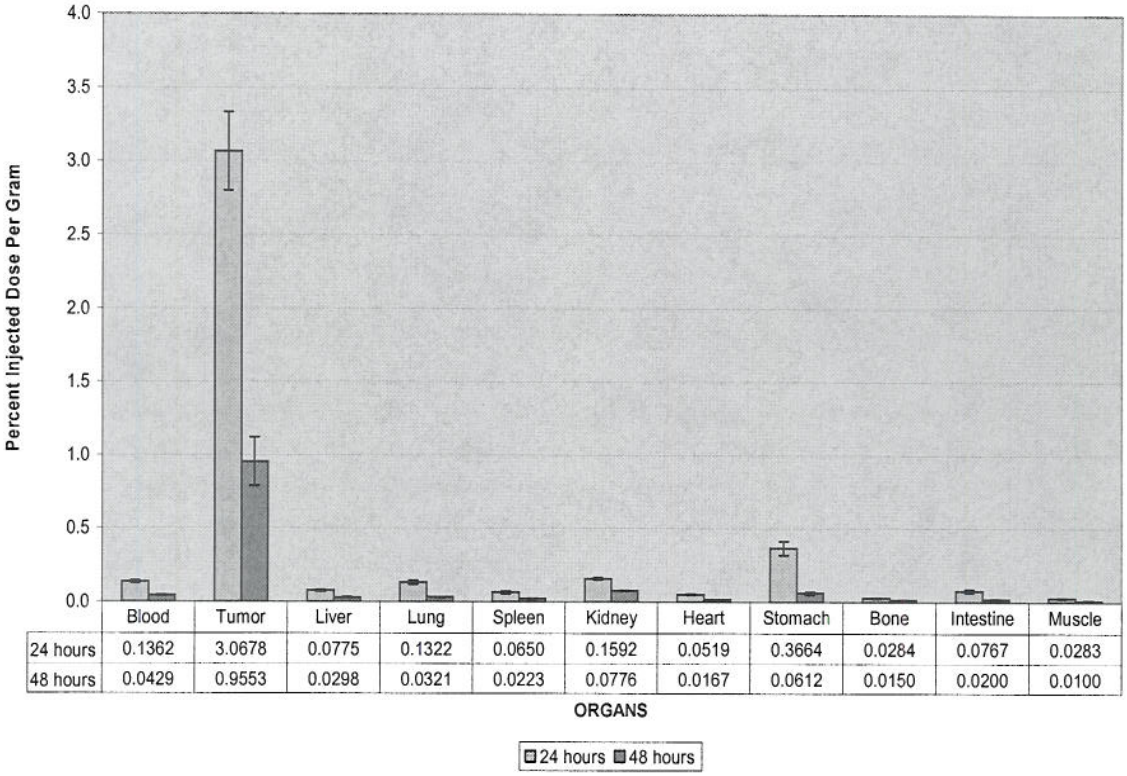


Effective targeting of BT474 xenografts with Anti-HER2 antibody

BT-474 breast cancer cells overexpress HER2, and maintain this HER2 overexpression when xenografted onto immunodeficient mice (data not shown). Biodistribution studies of ^{125}I-labeled antibodies targeting HER2 were performed. Biodistribution analyses were

performed using ALM (anti-HER2) antibody radiolabeled with ^{125}I using Iodogen. Cohorts of 5 scid mice bearing BT-474 xenografted tumors received 20 μCi per mouse of radioiodinated ALM via tail vein injection. Percent injected dose per gram of tissue (%ID/g) and cumulative tumor: organ ratios was determined by necropsy at 24 and 48 hours post injection as previously described [5]. As shown in **Figure 4** below, effective targeting of the anti-HER2 antibody was seen *in vivo* of BT-474 xenografts with tumor: organ ratios of > 20:1.

Figure 4. Biodistribution of I125-ALM in BT-474 containing scid mice

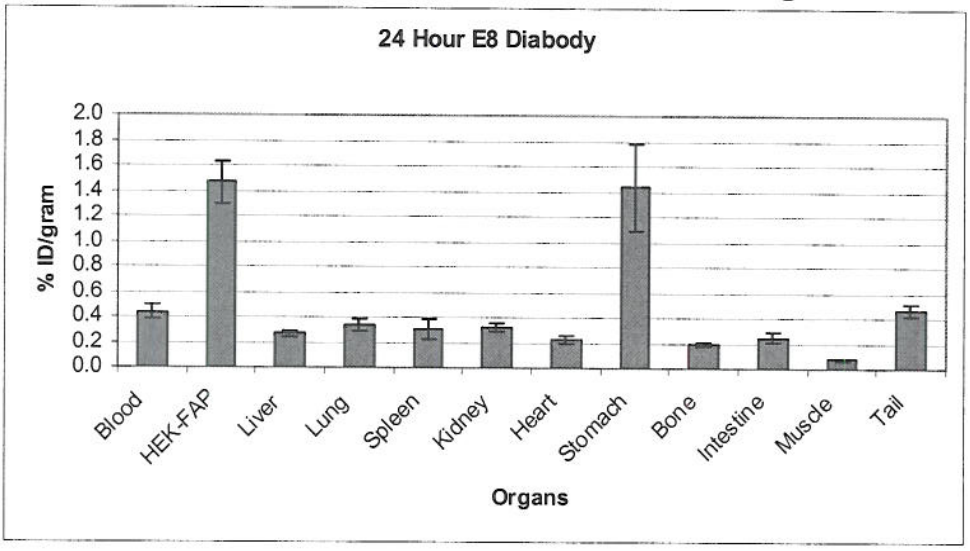


	Blood	Tumor	Liver	Lung	Spleen	Kidney	Heart	Stomach	Bone	Intestine	Muscle
24 hours	0.1362	3.0678	0.0775	0.1322	0.0650	0.1592	0.0519	0.3664	0.0284	0.0767	0.0283
48 hours	0.0429	0.9553	0.0298	0.0321	0.0223	0.0776	0.0167	0.0612	0.0150	0.0200	0.0100

Targeting of HEK-FAP xenografts with Anti-FAP antibody

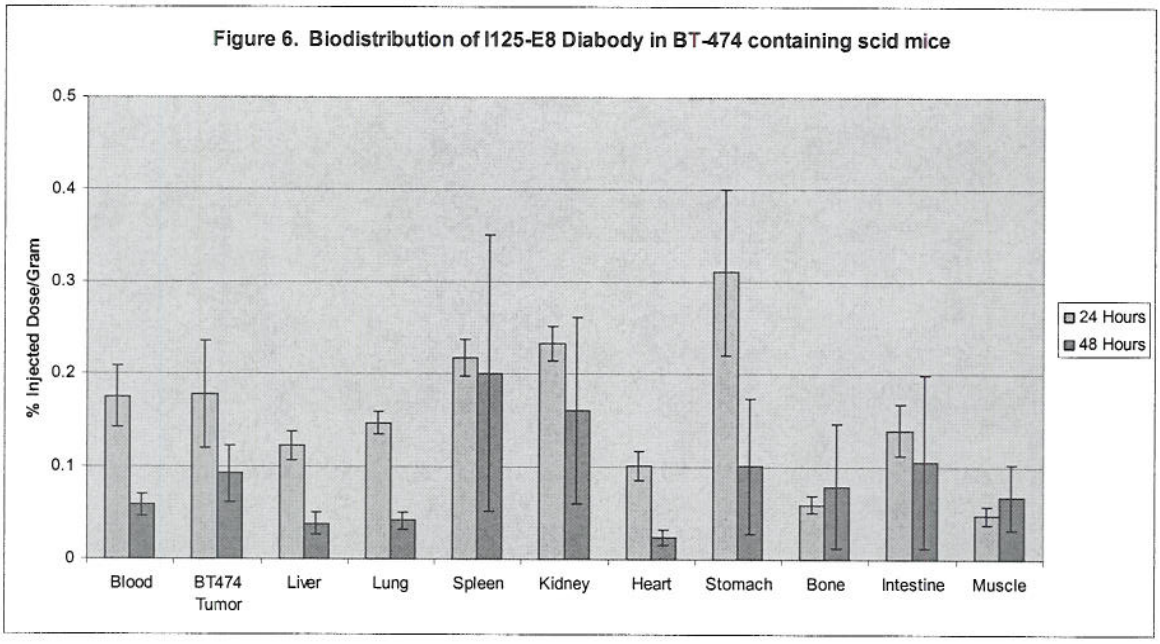
To demonstrate *in vivo* proof-of-principle targeting of the anti-FAP antibody E8, ^{125}I-labeled E8 diabodies were tested in a HEK-FAP xenograft model, whereby HEK293 tumor cells have been stably transfected to express FAP. As shown in **Figure 5** below, the E8 diabody can target FAP expressing HEK cells *in vivo*, with tumor:organ ratios of approximately 5:1. This confirms that the diabody E8 can target FAP *in vivo* in an animal tumor model.

Figure 5 Biodistribution of E8 diabody in HEK-FAP xenografts



Unsuccessful targeting of BT-474 xenografts with anti-FAP antibodies

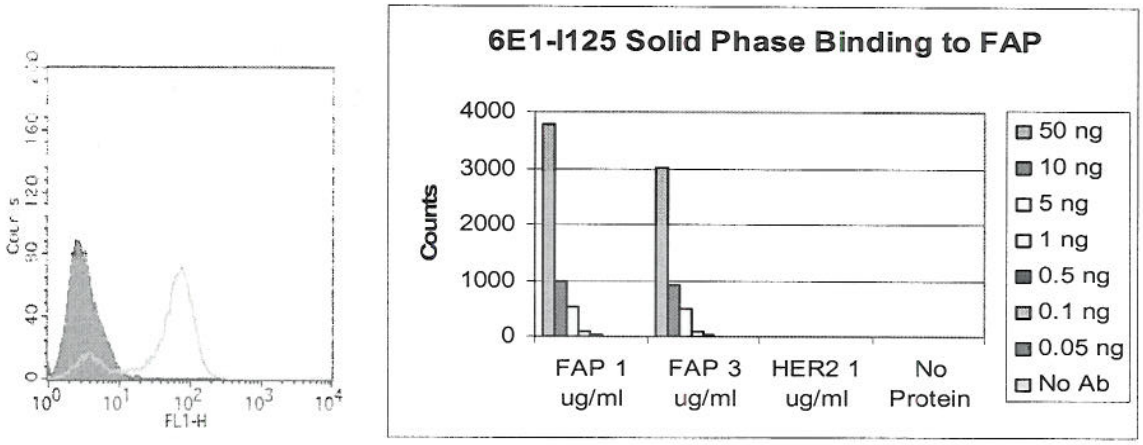
BT-474 breast cancer cells when xenografted onto nude mice induce stromal FAP expression on the stromal fibroblasts as shown in **Figure 1, Left Panel**. Biodistribution studies of ^{125}I-labeled E8 diabodies targeting FAP were performed using the methodology described above for the anti-HER2 antibody ALM. As shown in **Figure 6** below, E8 diabodies did not target stromal FAP *in vivo*, with a tumor: organ ratio of approximately 1.



To independently confirm these results, we performed a biodistribution studies using an anti-FAP IgG monoclonal antibody to exploit the relative stability of IgGs in the labeling process, and the predictable *in vivo* pharmacokinetics. As shown in **Figure 7, Left Panel**, the rabbit monoclonal antibody 6E1 effectively binds to HEK-FAP cells as analyzed by

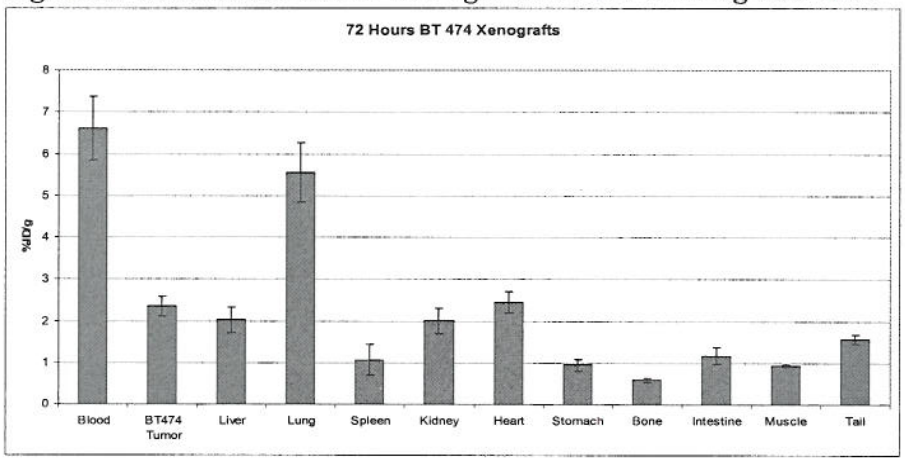
flow cytometry. Live cell binding assay of ^{125}I labeled 6E1 antibody demonstrates 30% binding to HEK-FAP cells (data not shown), and solid phase assessment of radiolabeled 6E1 given in decreasing concentrations shows effective targeting of recombinant FAP-ECD (**Figure 7**). Thus the rabbit monoclonal antibody 6E1 which targets murine FAP was assessed in a biodistribution study in BT-474 xenografts.

Figure 7. Binding of rabbit monoclonal 6E1 to murine FAP



Biodistribution analyses was performed using 6E1 (anti-FAP) antibody radiolabeled with ^{125}I using Iodogen (20 μCi per mouse) in cohorts of 5 scid mice bearing BT-474 xenografted tumors. Percent injected dose per gram of tissue (%ID/g) and cumulative tumor: organ ratios was determined by necropsy 72 hours post injection. Unfortunately the biodistribution studies using I^{125} radiolabeled 6E1 to target stromal FAP in BT-474 xenografts did not show clear tumor targeting as shown in **Figure 8** below.

Figure 8. Biodistribution of 6E1 IgG in BT474 bearing scid mice



There are a number of possibilities to explain the lack of antibody targeting of stromal FAP. These include:

1. Disruption of the antibody binding to FAP through the I125 labeling process
2. Degradation of the radiolabeled antibody *in vivo*
3. Poor penetration of the antibody into the tumor mass

4. Inadequate binding of antibody *in vivo* to FAP
5. Inadequate FAP expression on the stromal fibroblasts

Given the similar findings of lack of *in vivo* targeting by both antibody moieties E8 diabody and 6E1 IgG for BT474 xenografts, one can conclude that there is no advantage to targeting stromal FAP using the antibodies tested. The anti-HER2 antibody ALM does bind significantly to the target cancer cells, however there was inferior tumor:blood and tumor:organ ratios when FAP was targeted in the stromal compartment. Future studies may further explore stromal binding using higher affinity antibodies which may overcome some of the obstacles to stromal targeting, including the relatively low antigen density which may be present in the stroma.

KEY RESEARCH ACCOMPLISHMENTS

- Identification of BT474 breast cancer xenografts that induce strong stromal expression of FAP.
- Identification of scFv antibodies that bind with K_d 6.77e-9 affinity to murine FAP. This E8 antibody also binds to FAP in its native conformation on HEK293-FAP cells.
- Synthesis of anti-FAP E8 diabodies that bind with 6.88e-10 K_d affinity to murine FAP. This E8 diabody also binds FAP in its native conformation on HEK293-FAP cells.
- Effective targeting *in vivo* by anti-HER2 antibody ALM to BT-474 xenografts, which overexpress HER2.
- Effective targeting *in vivo* by anti-FAP antibody E8 diabody to HEK293-FAP xenografts.
- Lack of effective *in vivo* targeting by E8 diabodies to BT-474 xenografts that induce stromal FAP expression.
- Identification of rabbit monoclonal antibody 6E1 that retains its affinity for murine FAP after radiolabeling.
- Lack of effective *in vivo* targeting by 6E1 IgG to BT-474 xenografts that induce stromal FAP expression.

REPORTABLE OUTCOMES

Publications

- Cheng JD, Simmons H, Horak E, Dunbrack R, Valianou M, Alpaugh RK, and Weiner LM. Generation of antibodies targeting murine fibroblast activation protein

(FAP), a tumor stromal fibroblast protease. Proc Am Assoc Cancer Res 2001;42:A5094.

- Cheng JD, Valianou M, Canutescu AA, Jaffe EK, Lee HO, Wang H, Lai JH, Bachovchin WW, Weiner LM. Abrogation of fibroblast activation protein enzymatic activity attenuates tumor growth. Mol Cancer Ther. 2005 Mar;4(3):351-60. (describes 6E1 antibody)

- Narra K, Lee HO, Lerro A, Valvardi J, Azeez O, Jesson M, Aziz N, Jones B, Cheng JD. Inhibitors of the Stromal Protease Fibroblast Activation Protein Attenuate Tumor Growth *in vivo* Proc Am Assoc Cancer Res 47:A4382, 2006.

Presentations

- "Stromal antibody therapy" Fox Chase Cancer Center Research Conference, February 8, 2005

- "Fibroblast Activation Protein as a Therapeutic Target", Fox Chase Cancer Center Research Conference, May 9, 2006

Personnel

Jonathan Cheng, MD	Principal Investigator
Jiping Zhang, PhD	Post-doctoral Associate

CONCLUSION

Human breast cancers induce expression of FAP in the surrounding stromal fibroblasts to potentiate tumor growth and invasion. This research project proposed using engineered antibody fragments to deliver a cytotoxic payload to these tumor stromal fibroblasts, thus killing the breast stromal fibroblasts that support the growth of breast cancer. We have identified an appropriate animal model that allows for evaluation of both stromal and epithelial targeting of BT-474 xenografts. Although epithelial targeting was accomplished, stromal targeting of FAP remains under development. There was no advantage seen to targeting the tumor stroma using the antibodies tested in this proposal. We hypothesize that this may be due to the relatively lower antigen density in the tumor stroma compared to the tumor antigen HER2. Additional higher affinity antibody reagents may be needed to investigate a stromal radioimmunotherapeutic strategy to inhibit breast cancer growth in an animal model. If an effective radioimmunotherapy strategy to treat breast cancers in an animal model can be accomplished, this may lead to a novel therapeutic approach targeting the tumor stroma in patients with breast cancer.

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APPENDICES

- Cheng JD, Simmons H, Horak E, Dunbrack R, Valianou M, Alpaugh RK, and Weiner LM. Generation of antibodies targeting murine fibroblast activation protein (FAP), a tumor stromal fibroblast protease. Proc Am Assoc Cancer Res 2001;42:A5094.

- Cheng JD, Valianou M, Canutescu AA, Jaffe EK, Lee HO, Wang H, Lai JH, Bachovchin WW, Weiner LM. Abrogation of fibroblast activation protein enzymatic activity attenuates tumor growth. Mol Cancer Ther. 2005 Mar;4(3):351-60. (describes 6E1 antibody)

- Narra K, Lee HO, Lerro A, Valvardi J, Azeez O, Jesson M, Aziz N, Jones B, Cheng JD. Inhibitors of the Stromal Protease Fibroblast Activation Protein Attenuate Tumor Growth *in vivo* Proc Am Assoc Cancer Res 47:A4382, 2006.

proteolysis catalyzed by serine proteinases such as plasmin. Thus, GPI-PLD expression appears to correlate with tumor progression and regulates malignant properties of tumor cells in vitro.

#5094 Generation of Antibodies Targeting Murine Fibroblast Activation Protein (FAP), a Tumor Stromal Fibroblast Protease. Jonathan D. Cheng, Heidi Simmons, Eva Horak, Roland Dunbrack, Matthildi Valianou, R. Katherine Alpaugh, and Louis M. Weiner. *Fox Chase Cancer Center, Philadelphia, PA.*

Fibroblast activation protein (FAP) is a type II integral membrane glycoprotein belonging to the serine protease family. Human FAP is selectively expressed by tumor stromal fibroblasts in epithelial carcinomas, but not by epithelial carcinoma cells, normal fibroblasts, or other normal tissues. FAP has been shown to have both in vitro dipeptidyl peptidase and collagenase activity, but its biologic function in the tumor microenvironment is unknown. Although human FAP has been validated as a clinical target, animal models and antibodies targeting murine FAP remain to be developed. We have modeled the structure of murine FAP and shown it to consist of a short cytoplasmic tail, a single hydrophobic transmembrane region, and a large extracellular domain. The extracellular domain consists of 3 domains, a wrap-around domain, a 7 bladed β-propeller domain, and an $\alpha\beta$ hydrolase domain containing the catalytic triad, which is located in the central portion of FAP. The 7 bladed β-propeller domain situated on top of the catalytic triad may serve as a "gate" to selectively funnel proteins to the catalytic site. We have cloned murine FAP cDNA by RT-PCR of primary cultures of mouse embryonic fibroblasts. Murine FAP was expressed in a mammalian system using 293 (HEK) cells. Recombinant murine FAP has dipeptidyl peptidase activity in a fluorogenic assay using Ala-Pro-AFC as a substrate. We have also immunized rabbits and mice with the extracellular domain (ECD) of murine FAP. Rabbit sera containing polyclonal anti-FAP antibodies can inhibit murine FAP dipeptidyl peptidase activity. Mouse spleens were fused with SP/2 myeloma cells to produce hybridomas. Murine monoclonal antibodies targeting murine FAP-ECD have been produced. Such antibodies may be useful in exploring tumor-stromal interactions, and delineating FAP biology in an appropriate animal model.

Abrogation of fibroblast activation protein enzymatic activity attenuates tumor growth

Jonathan D. Cheng,[1] Matthildi Valianou,[1] Adrian A. Canutescu,[3] Eileen K. Jaffe,[3] Hyung-Ok Lee,[1] Hao Wang,[2] Jack H. Lai,[4] William W. Bachovchin,[4] and Louis M. Weiner[1]

Departments of [1]Medical Oncology and [2]Biostatistics, [3]Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania; and [4]Department of Biochemistry, Tufts University School of Medicine, Boston, Massachusetts

Abstract

Tumor-associated fibroblasts are functionally and phenotypically distinct from normal fibroblasts that are not in the tumor microenvironment. Fibroblast activation protein is a 95 kDa cell surface glycoprotein expressed by tumor stromal fibroblasts, and has been shown to have dipeptidyl peptidase and collagenase activity. Site-directed mutagenesis at the catalytic site of fibroblast activation protein, $Ser^{624} \rightarrow Ala^{624}$, resulted in an ~100,000-fold loss of fibroblast activation protein dipeptidyl peptidase (DPP) activity. HEK293 cells transfected with wild-type fibroblast activation protein, enzymatic mutant (S624A) fibroblast activation protein, or vector alone, were inoculated subcutaneously into immunodeficient mouse to assess the contribution of fibroblast activation protein enzymatic activity to tumor growth. Overexpression of wild-type fibroblast activation protein showed growth potentiation and enhanced tumorigenicity compared with both fibroblast activation protein S624A and vector-transfected HEK293 xenografts. HEK293 cells transfected with fibroblast activation protein S624A showed tumor growth rates and tumorigenicity potential similar only to vector-transfected HEK293. *In vivo* assessment of fibroblast activation protein DPP activity of these tumors showed enhanced enzymatic activity of wild-type fibroblast activation protein, with only baseline levels of fibroblast activation protein DPP activity in either fibroblast activation protein S624A or vector-only xenografts. These results indicate that the enzymatic activity of fibroblast activation protein is necessary for fibroblast activation protein–driven tumor growth in the HEK293 xenograft model system. This establishes the proof-of-principle that the enzymatic activity of fibroblast activation protein plays an important role in the promotion of tumor growth, and provides an attractive target for therapeutics designed to alter fibroblast activation protein–induced tumor growth by targeting its enzymatic activity. [Mol Cancer Ther 2005;4(3):351–60]

Introduction

Tumor-associated fibroblasts are functionally and phenotypically distinct from normal fibroblasts that are not in the tumor microenvironment. Tumor fibroblasts synthesize a wide variety of factors that can potentiate tumor growth and invasion. Fibroblasts normally provide mechanical support for tissue through the synthesis of extracellular matrix proteins. Fibroblasts in the tumor stroma have also been shown to express and secrete a large variety of proteases including stromelysin-3, plasminogen activator, and collagenase IV (1–3). Although some malignant cells can also produce proteases, the majority of protease production derives from the host stroma rather than from the tumor cells themselves (4). In addition to proteases, tumor stromal fibroblasts also play a critical role in the tumor microenvironment by providing growth factors and adhesion molecules that contribute to tumor invasion and motility (5–8). The mechanistic pathways by which tumor stromal fibroblasts contribute to the invasive and metastatic behavior of malignant cells are starting to be understood. The selectivity of fibroblast activation protein expression in the tumor stroma suggests that it may play a significant role in stromal contributions to tumor growth and invasion.

Fibroblast activation protein is a type II integral membrane glycoprotein belonging to the serine protease family. Fibroblast activation protein is a 95 kDa cell surface glycoprotein expressed by tumor stromal fibroblasts. Fibroblast activation protein has been shown to have dipeptidyl peptidase (DPP) and collagenase activity (9, 10). Fibroblast activation protein is well expressed by reactive stromal fibroblasts in >90% of human epithelial carcinomas (breast, lung, colorectal, and ovary) as determined by immunohistochemistry (11). Neural and lymphoid cells, as well as surrounding normal tissue, do not express fibroblast activation protein. Fibroblast activation protein expression in normal tissue has only been seen in a subset of pancreatic endocrine cells, and transiently in healing wounds. Consistent with its expression in areas of wound-healing fibrosis, fibroblast activation protein has been seen in the tissue-remodeling interface in areas of chronic inflammation, such as liver cirrhosis (12). Epithelial

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carcinoma cells are also fibroblast activation protein–negative, making fibroblast activation protein an attractive target for the study of tumor stromal cell biology. Stromal cells residing either in benign or premalignant epithelial breast fibroadenomas, or in colorectal adenomas are also predominantly fibroblast activation protein–negative, suggesting that malignancy is required for effective fibroblast activation protein induction. Some bone and soft tissue sarcomas are known to occasionally express fibroblast activation protein (13), consistent with fibroblast activation protein's mesenchymal origin. Seprase, which has a nearly identical genetic sequence to fibroblast activation protein (14), has been reported to be expressed in both the stromal and epithelial compartments of cancers (15–18), and may represent an isoform of fibroblast activation protein. However, the selectivity of fibroblast activation protein for tumor fibroblasts, but not epithelial tumor cells, has been confirmed by immunohistochemical staining in patients with colorectal cancer (10, 19), patients with breast cancer (9), and by reverse transcriptase-PCR analysis of pancreas, lung, and renal cell xenografts (20). Human and murine fibroblast activation proteins have a similar functional homology and an 89% shared sequence identity.

We have previously shown that forced fibroblast activation protein overexpression by tumor cells results in increased tumorigenicity and a significant enhancement in tumor growth (21). Here, we extend those observations by demonstrating that the enzymatic activity of fibroblast activation protein confers this tumor growth advantage. We show that enzymatic mutants of fibroblast activation protein that are devoid of fibroblast activation protein enzymatic activity, when xenografted into immunodeficient mice, result in attenuated tumor growth compared with the growth seen when tumors are transfected with wild-type fibroblast activation protein. In addition, treatment of mice with boronic acid inhibitors of fibroblast activation protein enzymatic activity results in tumor growth attenuation in a tumor model characterized by murine fibroblast activation protein expression in the surrounding tumor stromal fibroblasts. We thus provide the initial demonstration that specific inhibition of fibroblast activation protein enzymatic activity results in a therapeutic intervention by which tumor growth is attenuated.

Materials and Methods

Modeled Protein Structure of Murine Fibroblast Activation Protein

The murine fibroblast activation protein extracellular domain was modeled from the crystal structure of DPP IV (DPPIV) in the Protein Data Bank (22). PSI-BLAST (23) was used to search the nonredundant protein sequence database to construct a position-specific similarity matrix for the sequence of fibroblast activation protein. We searched a sequence database of proteins in the Protein Data Bank using the position-specific matrix, and chose to use the human DPPIV Protein Data Bank entries 1N1M and 1NU8 (to model the inhibitor) as templates, the alignment having an expectation value of 0.0 and a sequence identity of 53% over 722 amino acids of fibroblast activation protein (residues 38-759). This covered all eight blades of the propeller and the hydrolase domain. Side chain conformations for fibroblast activation protein were built onto the backbone structure of DPPIV with the program SCWRL (24), keeping conserved amino acids fixed in their crystallographic Cartesian coordinates. Loopy (25) was used for loop modeling. The software package Chimera[5] (26) was used for molecular graphics.

HEK293 Xenografts

Murine fibroblast activation protein cDNA was obtained from primary mouse embryonic fibroblast cultures as previously described (21). Fibroblast activation protein wild-type DNA was enzymatically mutated by site-directed mutagenesis of Ser^{624} to Ala^{624}. Mutagenic primer 5′-GCC-ATATGGGGCTGGGCCTACGGAGG-3′ were designed to flank the fibroblast activation protein DNA template and alter serine (TCC) at 624 to alanine (GCC). PCR amplification of the mutant DNA was engineered by annealing the mutagenic primers to parental fibroblast activation protein DNA template using QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. *Dpn*I restriction enzyme was added for digestion of parental, nonmutated DNA. The mutant clone S624A was transformed into XL10-Gold ultracompetent cells by heat pulsation at 42° for 30 seconds. The enzymatically mutated fibroblast activation protein was cloned into pSec/Hygro B plasmid, and expressed by HEK293 cells under antibiotic selection pressure. C.B17/Icr-severe combined immunodeficient (*scid*) mice were injected s.c. with 7×10^6 HEK293 cells transfected with fibroblast activation protein wild-type, fibroblast activation protein S624A, or vector alone. Animals were maintained under pathogen-free conditions in autoclaved microisolator cages in the Fox Chase Cancer Center Laboratory Animal Facility. Serial tumor measurements were obtained every 3 to 4 days by caliper in three dimensions. Tumor volumes were calculated by the formula volume = height × weight × length × 0.5236. Animals were followed until any mouse developed a tumor measuring > 2 × 2 cm, was observed to be suffering, or seemed moribund. Animals were euthanized according to institutional policy.

In vivo Assay of Fibroblast Activation Protein Dipeptidyl Peptidase Activity

This assay was done as described previously (21), with the following modifications. To determine the degree of fibroblast activation protein enzymatic activity in tumors, an immunocapture assay was done with Ala-Pro-7-amido-4-trifluoromethylcoumarin (Ala-Pro-AFC) as a substrate. Ninety-six-well Fluoronunc MaxiSorb plates were coated overnight at 4°C with anti–fibroblast activation protein rabbit polyclonal antibody obtained as previously described (21) at a 100 μg/mL dilution. Plates were rinsed with wash buffer consisting of PBS and 0.1% Tween 20, and

[5] http://www.cgl.ucsf.edu/chimera

blocked with 5% bovine serum albumin for 1 hour at room temperature. HEK293 or HT-29 xenografts were excised and proteins extracted using the detergent tissue protein extraction reagent (Pierce, Rockford, IL) according to the manufacturer's instructions. Tumors were homogenized for 60 seconds in the tissue protein extraction reagent at a concentration of 10 mL/g tumor, and samples centrifuged at 14,000 rpm × 10 minutes to pellet the cellular debris. Protein concentrations were assessed and normalized by spectrophotometric determination and bicinchoninic acid assay (Pierce). One milligram of the total tumor protein extracts, which contained fibroblast activation protein, was added to the wells and incubated for 1 hour, washed 10 times with PBS 0.1% Tween 20, and DPP activity assessed by cleavage of 0.25 mmol/L Ala-Pro-AFC for 1 hour at room temperature. Release of the free AFC fluorescent substrate was detected on a cytofluor fluorimeter (Labsystems, Helsinki, Finland) with 396 nm excitation and 490 emission.

Fibroblast Activation Protein Activity Assay

K_m and V_{max} values for fibroblast activation protein were estimated from data that measured the release of free AFC from the Ala-Pro-AFC substrate after incubation with fibroblast activation protein. The extracellular domain of both wild-type fibroblast activation protein and S624A fibroblast activation protein were recombinantly expressed, as previously described (21). Assays containing concentrations of 0.1, 0.25, 0.5, 1, and 5 mmol/L of the Ala-Pro-AFC substrate were done in triplicate using serial concentrations of fibroblast activation protein (100, 200, 1,000, 2,000, and 5,000 ng). Assays were done in 100 mmol/L Tris, 100 mmol/L NaCl (pH 7.8) at room temperature with reaction times varying from 5 minutes to 1 hour for wild-type fibroblast activation protein, and required extension of up to 24 hours in order to see product formation with the fibroblast activation protein S624A. Selecting only that data which estimated the initial rate and was within the linear region of the spectrofluorimeter, K_m and V_{max} values were determined by fitting a hyperbolic equation to the rate versus substrate concentration data using the program SigmaPlot (Statistical Package for the Social Sciences, Chicago, IL).

Immunohistochemistry

As previously described (21), HEK-fibroblast activation protein tumors from C.B17/Icr-*scid* mice were paraffin-embedded after fixation with 10% formaldehyde. Five-micron sections were cut and stained. Primary antibody consisting of SAS cut rabbit polyclonal anti–fibroblast activation protein antisera was used in a 1:750 dilution (v/v). Amplification and development by BioGenex (San Ramon, CA) biotin-streptavidin detection system with horseradish peroxidase was done as per manufacturer's directions. The pattern of fibroblast activation protein expression in the tumor sections was assessed.

Flow Cytometry

HEK293 cells transfected with either wild-type fibroblast activation protein or S624A fibroblast activation protein were analyzed by flow cytometry using the rabbit polyclonal anti–fibroblast activation protein antibodies. HEK293 cells (1×10^6) were aliquoted into Falcon 12 × 75 mm polystyrene tubes and the HEK293-transfected cells were washed in fluorescence-activated cell sorting buffer, and 5 μg of the rabbit polyclonal anti–fibroblast activation protein antibodies were added for 30 minutes. After washing again with fluorescence-activated cell sorting buffer, the secondary antibody of GAR-FITC was added for 30 minutes, cells fixed with 1% paraformaldehyde, and collected on a FACScan instrument (Becton Dickinson). MOPC-21 was used as an irrelevant control antibody. The entire procedure was done on ice.

Immunofluorescence

HEK293 cells (1×10^6) transfected with either wild-type fibroblast activation protein or S624A fibroblast activation protein were cultured on a cover slip overnight. HEK293-transfected cells were washed in PBS, then fixed using PBS containing 5% sucrose and 4% paraformaldehyde for 20 minutes. After rehydration in PBS, the cells were blocked for 1 hour with 10% bovine serum albumin and then washed in PBS. The cells were subsequently incubated for 60 minutes with 10 μg/mL of the primary rabbit monoclonal antibody 6E1, washed thrice, and incubated for 30 minutes with 3 μg/mL of the secondary rhodamine red donkey anti-rabbit F(ab′)$_2$ (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS with 3% bovine serum albumin. The cells were washed thrice for 10 minutes in PBS, and once in distilled water. Cover slips were mounted with Vectashield (Vector Lab, Burlingame, CA) with 4′,6-diamidino-2-phenylindole for nuclear staining.

Western Analyses

SDS-PAGE gels (10%) were loaded with 200 μg of tissue protein extraction reagent extracted proteins from HT-29 xenografts and transferred to a nitrocellulose membrane. After blocking with 10% milk in PBS + 0.1% Tween, the rabbit polyclonal antibody targeting murine fibroblast activation protein at a dilution of 20 μg/mL, and the secondary antibody of donkey anti-rabbit horseradish peroxidase (Amersham, Piscataway, NJ) at a 1:1,000 dilution (v/v) was used. Films were developed using Supersignal West Pico Chemiluminescent Substrate (Pierce). Anti-β-actin antibody (Sigma, St. Louis, MO) in a 1:1,000 dilution was used as a control of loaded protein.

In vitro and *In vivo* Assay of Val-boroPro

Val-boroPro is a boronic acid inhibitor of fibroblast activation protein enzymatic activity. To determine the pH dependence of Val-boroPro in inhibiting fibroblast activation protein enzymatic activity, Val-boroPro was incubated overnight either in 0.01 N HCl or neutral reaction buffer consisting of 100 mmol/L Tris, 100 mmol/L NaCl (pH 7.8). Serial 10-fold dilutions of Val-boroPro were incubated with 3 nmol/L recombinant murine fibroblast activation protein-extracellular domain at room temperature for 15 minutes prior to the addition of 0.25 mmol/L Ala-Pro-AFC substrate to give final inhibitor concentrations ranging from 4×10^{-4} to 4×10^{-12} mol/L. Inhibition of enzymatic activity was analyzed using a cytofluor fluorimeter and normalized as the percentage of baseline release of free AFC by fibroblast activation protein without inhibitor.

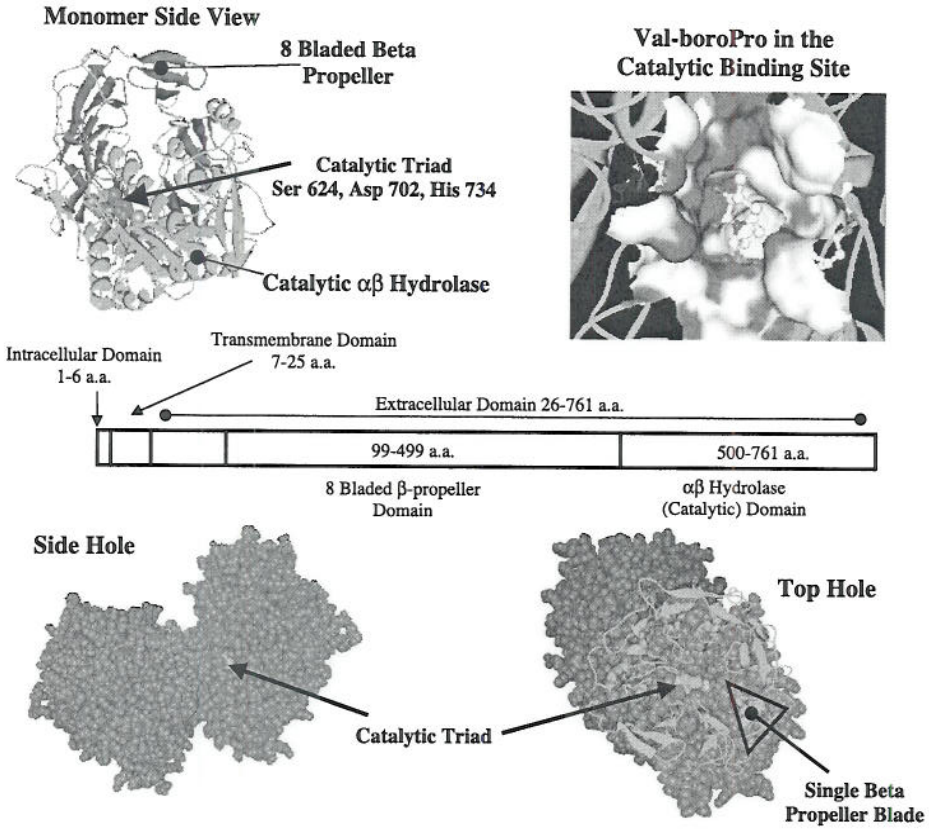

Figure 1. Predicted murine fibroblast activation protein structure. The modeled structure of fibroblast activation protein based on the crystal structure of DPPIV is hypothesized to consist of homodimers with a larger side hole of ~25 Å (*bottom left, Side Hole*), and a second opening in the β-propeller domain of ~10 Å (*bottom right, Top Hole*). The fibroblast activation protein inhibitor Val-boroPro is modeled interacting with Ser^{624} and His^{734} of the catalytic triad.

The *in vivo* effects of Val-boroPro on tumor growth were assessed in cohorts of five C.B17/Icr-*scid* mice inoculated with 5×10^6 HT-29 colorectal cancer cells s.c. in the flank. Mice were treated with either 50 μg of the fibroblast activation protein inhibitor Val-boroPro in 100 μL normal saline, or 100 μL normal saline by gastric gavage daily for 3 weeks (days 1-21 of inoculation). Animals were maintained under pathogen-free conditions in autoclaved microisolator cages in the Fox Chase Cancer Center Laboratory Animal Facility. Serial tumor measurements were obtained twice a week by caliper in three dimensions. Tumor volumes were calculated by the formula volume = height × weight × length × 0.5236. Animals were followed until any mouse developed a tumor measuring > 2 × 2 cm, was observed to be suffering, or seemed moribund. Animals were euthanized according to institutional policy.

Statistical Method

A random coefficient model was applied to model the change of tumor size over time and examine the treatment effect on tumor growth. The tumor size for each animal was modeled as a quadratic function over time. Welch's variance-weighted one-way ANOVA with correction for unequal variances was used to compare treatment groups with respect to tumor size at each time point. Repeated measure ANOVA approach was used to compare fluorescence measures of fibroblast activation protein enzymatic activity among treatment groups. All the statistical analyses were carried out using SAS software and P values less than 0.05 indicated statistical significance.

Results

Revised Model of Fibroblast Activation Protein

We have previously described the modeled structure of murine fibroblast activation protein based on the crystal structure of prolyl oligopeptidase, which shares a 12% sequence identify with fibroblast activation protein (21). The recent availability of the crystal structure of DPPIV (22), which shares a 53% sequence homology with fibroblast activation protein allowed the construction of a revised model of murine fibroblast activation protein as shown in Fig. 1. The modeled structure consists of a short cytoplasmic tail (amino acids 1-6), a single hydrophobic transmembrane region (amino acids 7-25), and a large extracellular domain (amino acids 26-761) consisting of two major domains: (*a*) bladed β-propeller domain (amino acids 99-499), and (*b*) αβ hydrolase domain (amino acids 500-761) that contains the catalytic triad. The catalytic triad consists of a serine at position 624, aspartic acid at 702, and histidine at 734. The availability of the DPPIV crystal structure allowed a complete modeling of the eight-blade β propeller component that was just predicted in the

previous model, as the closest template structure at that time was a seven-blade β propeller. In addition, using a newly available crystal structure (1NU8.pdb), we were also able to build a model containing the fibroblast activation protein inhibitor Val-boroPro in the conserved binding site as shown in Fig. 1. The model places the serine catalytic triad in close proximity to the dimerization motif, located within the eight β-propeller domain. The model predicts that site-directed mutation of $S^{624} \rightarrow A^{624}$ will induce loss of fibroblast activation protein DPP activity. The revised model also predicts that fibroblast activation protein forms homodimers with a larger side hole of ~25 Å, and a second smaller opening in the β-propeller domain of ~10 Å. Although there is a four-residue difference between DPPIV and fibroblast activation protein out of 28 side chains in the dimer interface ($T^{736} \rightarrow S^{730}$, $T^{746} \rightarrow R^{740}$, $G^{727} \rightarrow Q^{721}$ and $D^{725} \rightarrow N^{719}$), this resulted in replacement of a hydrogen bond (T^{746}-$D^{725} \rightarrow R^{740}$-$Q^{721}$), and is unlikely to influence the homodimerization interface stability of the modeled fibroblast activation protein.

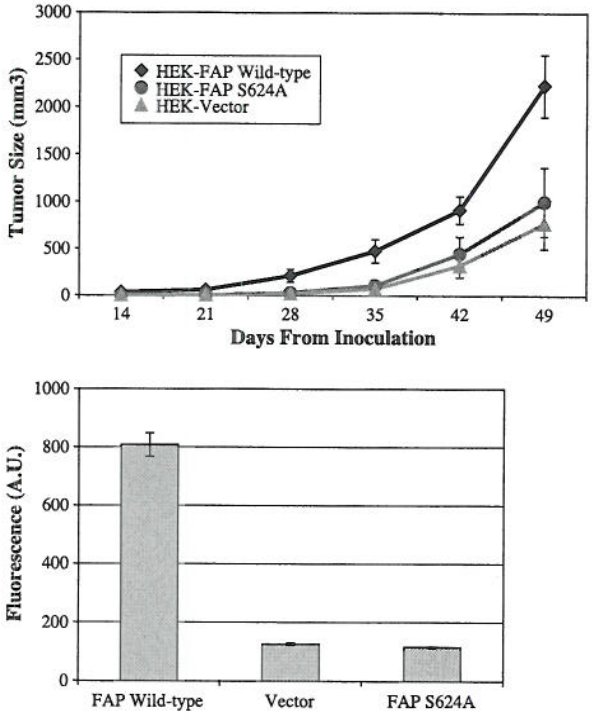

Figure 2. Potentiation of tumor growth by fibroblast activation protein transfection of HEK293 xenografts is dependent on its enzymatic activity. Tumor volumes were assessed weekly from the time of inoculation with 7×10^6 HEK293 cells transfected with either fibroblast activation protein wild-type (♦), fibroblast activation protein S624A (●), or vector-only (▲). Potentiation of tumor growth by HEK-fibroblast activation protein wild-type xenografts was seen ($P < 0.0001$, HEK-fibroblast activation protein wild-type versus HEK-fibroblast activation protein S624A). However, no statistically significant differences in tumor growth were seen between HEK-fibroblast activation protein S624A and HEK-vector. The bottom panel shows the *in vivo* fibroblast activation protein enzymatic activity of these xenografts. The DPP enzymatic activity of HEK293-fibroblast activation protein wild-type, HEK293-fibroblast activation protein S624A, and HEK293-vector xenografts was assessed on day 21 using an immunocapture assay of fibroblast activation protein from total protein lysates of these tumors. HEK293-fibroblast activation protein wild-type xenografts showed significantly enhanced fibroblast activation protein DPP activity over fibroblast activation protein S624A and vector controls ($P = 0.0031$ and $P = 0.0032$, respectively), but no significant differences in DPP activity were seen between HEK293-fibroblast activation protein S624A and HEK293-vector xenografts ($P = 0.133$).

Fibroblast Activation Protein Growth Potentiation Is Dependent on Its Enzymatic Activity

Serial substrate dilutions of Ala-Pro-AFC were incubated with recombinantly expressed fibroblast activation protein-extracellular domain, and the data fitted to the Michaelis-Menten equation yielding a wild-type fibroblast activation protein K_m of ~0.25 mmol/L, with a V_{max} of 55 μmol/L/minute/μg. In contrast, site-directed mutagenesis of the catalytic site of fibroblast activation protein S624A resulted in an enzymatic mutant with ~10,000- to 100,000-fold loss of fibroblast activation protein DPP activity at 1 mmol/L substrate concentration. Full-length fibroblast activation protein S624A was expressed by HEK293 cells. This enzymatically inactive mutant was utilized to assess the contribution of fibroblast activation protein enzymatic activity to tumor growth as compared with our previously described construct of wild-type fibroblast activation protein (21). Immunofluorescence and flow cytometry under nonpermeabilized conditions confirmed the cell surface presence of both wild-type and S624A fibroblast activation protein transfections of HEK293 cells (data not shown).

C.B17/Icr-*scid* mice were injected s.c. with HEK293 cells that had been transfected with wild-type, and S624A fibroblast activation proteins, or vector alone (mock), and serial tumor measurements were obtained. The growth potentiation of wild-type fibroblast activation protein compared with vector-transfected HEK293 xenografts was confirmed, as shown in the tumor growth curves for all 18 mice in each cohort (Fig. 2). Enhanced tumorigenicity of fibroblast activation protein overexpressing tumors was also seen, as all 18 mice inoculated with 7×10^6 HEK293-fibroblast activation protein wild-type developed tumors by day 49, when mice with large tumors were euthanized according to institutional policy. In contrast, only 13 of 18 and 11 of 18 mice inoculated with HEK293-fibroblast activation protein S624A or HEK293-vector only cells developed tumors at day 49, respectively ($P = 0.046$ comparing HEK-fibroblast activation protein versus HEK-fibroblast activation protein S624A). In addition, HEK293-fibroblast activation protein S624A xenografts showed tumor growth rates similar only to vector-transfected HEK293 controls (Fig. 2). These results indicate that the enzymatic activity of fibroblast activation protein is necessary for fibroblast activation protein-driven tumor growth in fibroblast activation protein transfected HEK293 cells.

In vivo determination of fibroblast activation protein DPP enzymatic activity in the tumor xenografts was assessed. Rabbit polyclonal antibodies were used to capture fibroblast activation protein contained within total protein lysates from fibroblast activation protein transfected HEK293 xenografts obtained 21 days after inoculation into the flanks of C.B17/Icr-*scid* mice. Significantly

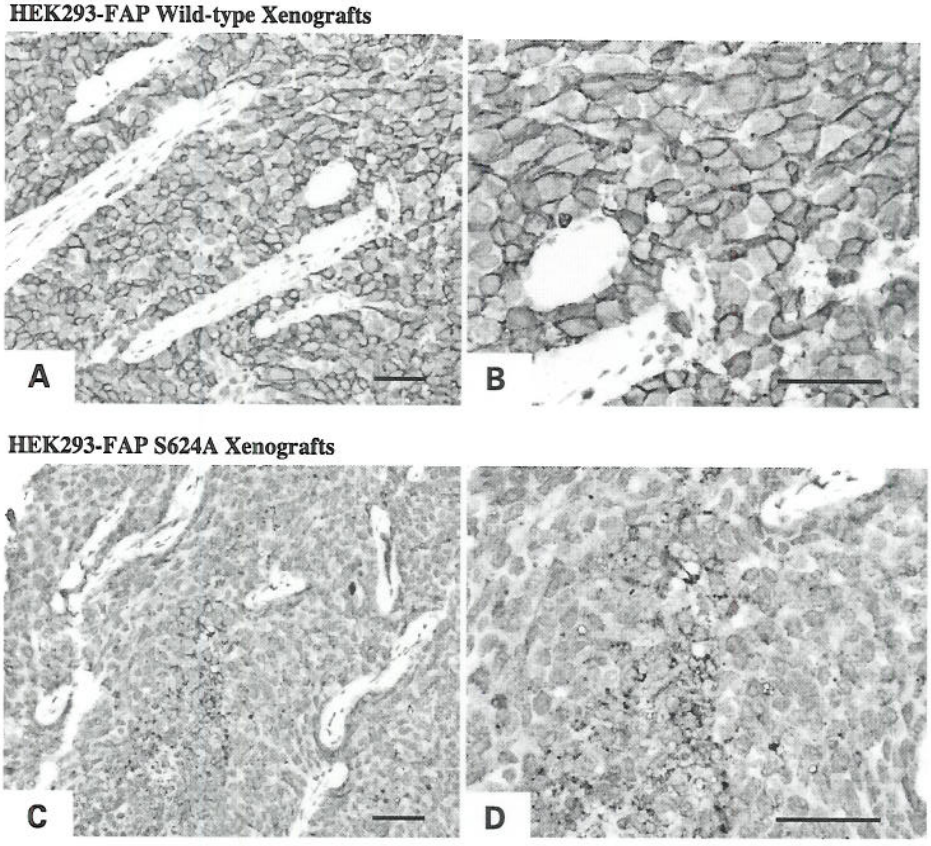

Figure 3. Immunohistochemistry of HEK293 xenografts transfected with wild-type or enzymatic mutant fibroblast activation proteins. Immunohistochemical analysis of HEK293-fibroblast activation protein wild-type and HEK293-fibroblast activation protein S624A xenografts stained with anti–fibroblast activation protein rabbit polyclonal antibodies shows the readily detectable presence of fibroblast activation protein wild-type on the cell membrane of transfected HEK293 cells (**A** and **B**, 20× and 40× magnification, respectively), but rare cytoplasmic staining of fibroblast activation protein S624A (**C** and **D**, 20× and 40× magnification, respectively). *Bar*, 50 μm.

enhanced DPP activity was seen in wild-type fibroblast activation protein compared with fibroblast activation protein S624A or vector only controls as seen in Fig. 2 (*bottom*). Thus, fibroblast activation protein–mediated growth potentiation of HEK293 tumor xenografts is associated with enhanced DPP enzymatic activity within tumors. These results extend our previous observations and support the premise that specific inhibition of fibroblast activation protein enzymatic activity may provide a therapeutic intervention by which tumor growth is attenuated.

Immunohistochemical Analyses of Xenografts

Immunohistochemistry studies done on the HEK293 xenografts showed cell surface membrane staining of the individual HEK293 cells transfected with wild-type fibroblast activation protein as seen in Fig. 3. Interestingly, immunohistochemical analysis of tumors obtained from mice inoculated with HEK293-fibroblast activation protein S624A showed infrequent fibroblast activation protein expression by the individual HEK293 cells. Furthermore, when fibroblast activation protein expression was seen in the HEK293-fibroblast activation protein S624A cells, it was cytoplasmic rather than on the cell membrane. This was surprising given the robust protein presence of fibroblast activation protein S624A on the surface of the inoculated HEK293 cells that was readily detected by both flow cytometry and immunofluorescence. Although HEK293-fibroblast activation protein S624A cells in culture can exhibit the presence of both cell surface and cytoplasmic fibroblast activation protein S624A (data not shown), the expression levels of fibroblast activation protein at the cell surface of HEK293 fibroblast activation protein wild-type and HEK293 fibroblast activation protein S624A cells are similar. However, in vivo inoculation of HEK fibroblast activation protein S624A cells results in greatly diminished fibroblast activation protein S624A expression and loss of cell surface localization (Fig. 3C and D). This suggests that the enzymatically inactive fibroblast activation protein not only fails to provide the growth advantage of wild-type fibroblast activation protein (as seen in Fig. 2), but that *in vivo* negative selection occurs with respect to both expression and localization of the enzymatically inactive protein.

Inhibition of Fibroblast Activation Protein Enzymatic Activity Is Associated with Growth Attenuation of HT-29 Xenografts That Induce Stromal Expression of Fibroblast Activation Protein

Recombinant murine fibroblast activation protein-extracellular domain was incubated with Val-boroPro prior to the addition of Ala-Pro-AFC as a fluorescent substrate. This yielded *in vitro* inhibition of fibroblast activation protein enzymatic activity with an IC_{50} of 4×10^{-8} mol/L

(pH 2) as shown in Fig. 4. This inhibition is pH-dependent, with a significant loss of inhibitory potency at neutral pH, as Val-boroPro remains in linear form at acidic pH, but cyclizes at neutral pH.

Xenografts of the HT-29 colorectal cancer cell line in C.B17/Icr-*scid* mice induce expression of fibroblast activation protein on host tumor stromal fibroblasts. The HT-29 cell line itself does not express fibroblast activation protein (data not shown), but induces the expression of fibroblast activation protein in the surrounding tumor stroma of HT-29 xenografts as we have previously shown (21). Western analysis using rabbit polyclonal antibodies confirms the expression of murine fibroblast activation protein in the HT-29 xenograft protein lysates. Figure 5 shows the *in vivo* tumor growth attenuation of HT-29 xenografts treated by gastric gavage with the fibroblast activation protein inhibitor Val-boroPro once daily for 21 consecutive days. On day 24 after inoculation, Val-boroPro-treated animals had a 45% reduction in HT-29 xenograft tumor size compared with normal saline–treated controls (459.0 ± 107.2 mm^3 versus 837.0 ± 39.7 mm^3, respectively). No obvious toxicities were observed in any of the animals treated with this boronic acid small molecule inhibitor of DPP activity. The degree of *in vivo* inhibition of fibroblast activation protein DPP activity at the tumor site after treatment with Val-boroPro was also assessed. Tumor protein extracts of HT-29 xenografts from mice treated for 7 days with 50 μg/day of Val-boroPro by gastric gavage were assessed for fibroblast activation protein enzymatic activity. Using an immunocapture assay of fibroblast activation protein from these tumors, a 44% inhibition of fibroblast activation protein enzymatic activity was seen in tumors treated with Val-boroPro compared with normal saline controls (105.5 ± 33.0 versus 194.2 ± 25.9 A.U., respectively; $P = 0.046$). Representative samples from three mice treated with Val-boroPro show similar expression levels of fibroblast activation protein compared with three control mice as shown in Fig. 5 (*bottom*). Thus, treatment with Val-boroPro altered the *in vivo* enzymatic activity, but not the level of fibroblast activation protein expression.

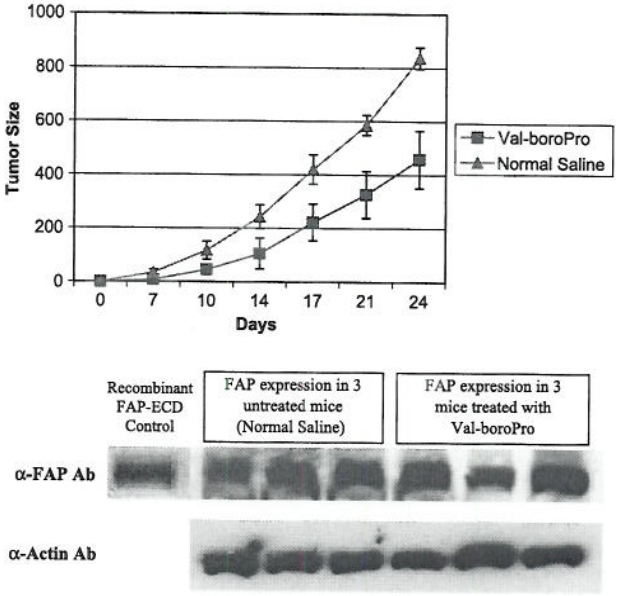

Figure 5. Attenuation of HT-29 xenograft tumor growth by small molecule inhibitor of fibroblast activation protein enzymatic activity. Tumor volumes of HT-29 colorectal cancer xenografts were assessed weekly in an animal model whereby fibroblast activation protein is induced in the mouse stroma as HT-29 cells themselves are negative for fibroblast activation protein expression. Tumor growth attenuation was seen in mice treated by gastric gavage with 50 μg of the fibroblast activation protein inhibitor Val-boroPro once daily for 21 consecutive days, $P = 0.0312$. Western analysis (*bottom*) of proteins extracted from HT-29 xenografts using rabbit polyclonal anti–fibroblast activation protein antibodies show similar levels of fibroblast activation protein expression in both treated and untreated tumors. A protein loading control as assessed by anti-actin antibody was used.

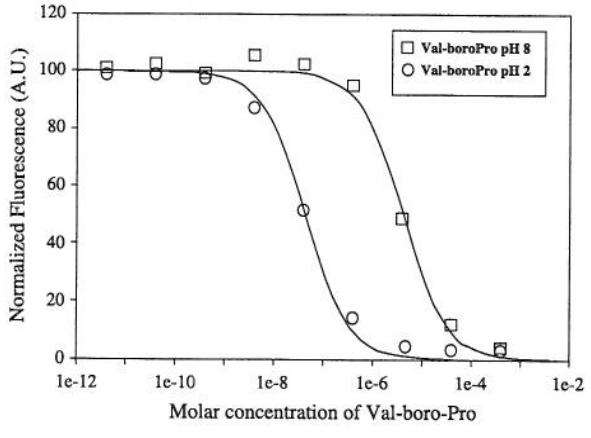

Figure 4. *In vitro* inhibition of fibroblast activation protein by Val-boroPro. The pH dependence of Val-boroPro in inhibiting fibroblast activation protein enzymatic activity is shown using serial dilutions of Val-boroPro in either 0.01 N HCl (pH 2) or neutral reaction buffer (pH 8). Inhibition of fibroblast activation protein DPP enzymatic activity by Val-boroPro was analyzed using a cytofluor fluorimeter and normalized as the percentage of baseline release (without inhibitor) of free AFC by fibroblast activation protein cleavage of the Ala-Pro-AFC substrate. A two-log greater potency of Val-boroPro in acidic pH was seen with a K_d of 4.35×10^{-8} and 4.16×10^{-6} mol/L for pH 2 and pH 8, respectively.

Therefore, Val-boroPro treatment inhibits fibroblast activation protein enzymatic activity *in vitro*, and attenuates tumor growth *in vivo*. In addition, pharmacodynamic assessment of fibroblast activation protein DPP activity *in vivo* of the HT-29 xenografts shows a reduction of fibroblast activation protein enzymatic activity at tumor sites, but not fibroblast activation protein expression levels following treatment by Val-boroPro. This was accomplished using a tumor model that recapitulates human epithelial cancer biology, with induction of fibroblast activation protein in the tumor stroma.

Discussion

The studies reported here show the proof-of-principle that inhibition of the DPP activity of fibroblast activation protein, a stromal selective protein, attenuates the growth

of epithelial carcinomas. This extends our previous observation that overexpression of fibroblast activation protein wild-type protein confers a tumor growth advantage by demonstrating that the enzymatic activity of fibroblast activation protein is responsible for this growth advantage. Transfected HEK293 cells were chosen for study in an animal model due to its modest tumor growth rates, and the ability of these cells to readily express transfected mammalian proteins. Although HEK293 cells are epithelial and not stromal in origin, overexpression of fibroblast activation protein in this model system provides the proof-of-principle of the role of fibroblast activation protein in tumor growth and invasion, and may be akin to clinical cancers characterized by overexpression of fibroblast activation protein in the tumor cell itself as seen in certain sarcomas (13). We also provide evidence of the role of fibroblast activation protein enzymatic activity in a stromal selective model of fibroblast activation protein using HT-29 colorectal cancer xenografts.

The mechanistic pathway(s) by which fibroblast activation protein potentiates tumor growth is not known, as natural substrates of fibroblast activation protein have not yet been identified. Given the dual enzymatic activity of fibroblast activation protein as both a DPP and a collagenase, multiple substrates with distinct biological effects may be found. It is tempting to speculate that fibroblast activation protein may degrade extracellular matrix via its collagenase activity, while regulating other hormones through its DPP cleavage of a penultimate alanine or proline. Intriguingly, seprase forms complexes with $\alpha_3\beta_1$ integrin in the presence of collagen (27), suggesting that fibroblast activation protein may promote an invasive phenotype through cell adhesion pathways. The dual specificity of fibroblast activation protein for both DPP and collagenase activity utilizes the same serine catalytic site (10), and thus can be targeted for therapeutic effect. Using an enzymatically inactive fibroblast activation protein mutant, we provide the first direct evidence that abrogation of fibroblast activation protein enzymatic activity *in vivo* results in tumor growth attenuation of fibroblast activation protein–driven growth.

We find that fibroblast activation protein overexpression potentiates tumor growth that is dependent on this protein's enzymatic activity. This contrasts with a recent report suggesting that fibroblast activation protein may be a tumor suppressor, through mechanisms that are independent of its enzymatic activity, Ramirez-Montagut et al. (28) reported that fibroblast activation protein reexpression by malignant melanoma cells abrogated tumorigenicity, and that this effect was independent of its enzymatic activity. In fact, the enzymatic mutant was a more potent tumor suppressor than wild-type fibroblast activation protein in this melanoma system. One possible explanation for such a disparate set of conclusions is that HEK293 cells are epithelial, whereas melanoma cells have mesenchymal features. Although melanocytes lose fibroblast activation protein expression with malignant transformation, expression of stromal fibroblast activation protein enzymatic activity is enhanced in the process of melanoma carcinogenesis (29). We speculate that a negative biological consequence for melanoma cells that express fibroblast activation protein leads to an induction or outsourcing of fibroblast activation protein functions to the surrounding stromal fibroblasts. Thus, normal physiologic processes of fibroblast activation protein that are seen in tadpole tail resorption and wound healing (11, 30, 31) may be usurped in the activated tumor stroma to promote an invasive tumor phenotype. This growth potentiation effect of fibroblast activation protein/seprase was recently independently confirmed in breast cancer (32).

Although the vast majority of epithelial malignancies induce expression of human fibroblast activation protein by tumor stromal fibroblasts, a small proportion of clinical epithelial tumors invade and metastasize independently of fibroblast activation protein. Clearly, factors other than fibroblast activation protein contribute to tumor invasion. Tumor growth independent of fibroblast activation protein expression is seen in fibroblast activation protein–negative tumors, both in the clinic and in the studies reported here. Other stromal factors that participate in tumor growth, or "the company fibroblast activation protein keeps," are the subject of ongoing investigations. We also cannot exclude the possibility that fibroblast activation protein may interact with other proteins that regulate tumor growth independent of its enzymatic activity. Tumor growth rates did not differ between the HEK293 xenografts transfected with either fibroblast activation protein S624A or the empty vector, although there was a slight trend favoring HEK293-fibroblast activation protein S624A compared with HEK293-vector xenografts. Despite these caveats, fibroblast activation protein enzymatic function provides a potentially important new therapeutic target that could be applicable to the therapy of a variety of human malignancies.

Dipeptides containing the α-amino boronic acid analogue of proline, boroPro, are potent small molecule inhibitors of DPP enzymatic activity (33, 34). These boronic acid compounds are reversible transition state analogues, demonstrating K_i values in the picomolar range (35), and have known *in vivo* biological effects (36), including antitumor effects (37). The studies reported here show the ability of Val-boroPro to not only inhibit fibroblast activation protein DPP enzymatic activity *in vitro*, but also partially inhibit fibroblast activation protein DPP enzymatic activity *in vivo*. This is accompanied by a modest growth attenuation of HT-29 tumors that induce fibroblast activation protein expression in the surrounding stroma. This association between inhibition of fibroblast activation protein enzymatic activity *in vivo* and tumor growth attenuation is consistent with the fibroblast activation protein S624A transfection experiments reported here, and supports our hypothesis that inhibition of fibroblast activation protein enzymatic activity attenuates the invasive capabilities of tumors.

Given the ability of Val-boroPro to inhibit the enzymatic activity of both fibroblast activation protein and DPPIV, the relative contributions to tumor growth of dual inhibition of fibroblast activation protein/DPPIV as opposed to fibroblast

activation protein–specific effects are unclear. However, a number of lines of evidence suggest that the tumor attenuation effects of Val-boroPro are due to inhibition of fibroblast activation protein and not DPPIV: (*a*) the biological effects of Val-boroPro on fibroblast activation protein can be seen independent of DPPIV as shown in DPPIV-deficient mice (36); (*b*) although there is heterodimerization of fibroblast activation protein and DPPIV in human cells, these proteins are uncoupled in mouse cells (28); (*c*) protein extracts of treated and untreated HT-29 xenografts did not show significant protein levels of DPPIV by Western analysis (data not shown). However, it is possible that Val-boroPro affects other currently unrecognized proteases that participate in tumor growth, and may have biological effects distinct from fibroblast activation protein. Future plans include further delineation of biological effects of fibroblast activation protein–specific inhibition utilizing fibroblast activation protein–selective small molecule or monoclonal antibody inhibitors of fibroblast activation protein. The specificity of monoclonal antibodies for fibroblast activation protein may offer significant advantages in determining fibroblast activation protein's specific contribution to tumor stromagenesis.

In summary, we have shown that fibroblast activation protein overexpression potentiates tumor growth, and abrogation of its enzymatic activity can attenuate fibroblast activation protein-driven tumor growth. This establishes the proof-of-principle that the enzymatic activity of fibroblast activation protein plays an important role in the promotion of tumor growth, and is an attractive target for therapeutics designed to alter fibroblast activation protein-induced tumor growth by targeting its enzymatic activity.

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97th AACR Annual Meeting
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Abstract Number: 4382

Presentation Title: **Inhibitors of the stromal protease fibroblast activation protein attenuate tumor growth in vivo**

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Location: Exhibit Hall, Washington Convention Center

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Poster Board Number: 30

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Fibroblast activation protein (FAP) is a serine protease that is selectively expressed on tumor stromal fibroblasts in a vast majority of epithelial cancers. We have previously shown that FAP overexpression enhances tumor growth and suggested a possible therapeutic role for functional inhibition of FAP activity (Cancer Res 62:4767-4772, 2002). PT-100 and PT-630 are boronic dipeptides, which competitively inhibit dipeptidyl peptidases including FAP and DPP-IV/CD26 with K_i in the nanomolar range. PT-100 treatment has been shown to decrease tumor size and cause tumor regression and rejection in a variety of animal tumor models (Cancer Res 64: 5471-5480, 2004). Its mechanism of action is complex, as it not only inhibits FAP enzymatic activity but also has been shown to have immunomodulatory effects including upregulation of cytokine and chemokine expression. PT-630 inhibits FAP enzymatic activity to a similar degree as PT-100, but without invoking a cytokine response. Therefore, we investigated the effects of PT-630 and PT-100 in animal models that have strong FAP expression. HT-29 xenografts induce stromal FAP expression in the tumor fibroblasts without any detectable expression on the colorectal cancer cells themselves. In addition HEK293 cells transfected with FAP were also tested as an epithelial FAP overexpression animal model. PT-100 and PT-630 when administered orally to mice inoculated subcutaneously with either HT-29 or HEK-FAP cells, significantly inhibited tumor growth when compared to saline treated controls. Both PT-100 and PT-630 decreased tumor volume in the HT-29 and HEK-FAP xenografts by approximately 50%. Immunocapture of FAP and DPP-IV from the tumors showed significantly decreased enzymatic activity in PT 100 and PT 630 treated xenografts indicating that FAP activity is suppressed in vivo. A dose related response for PT-630 was also suggested. We therefore demonstrate that PT-100 and PT-630 inhibit tumor growth of HT-29 and HEK-FAP xenografts. In addition, PT-100 and PT-630 attenuate enzymatic activity in vivo in a dose dependent manner. Given that PT-630 has minimal immunomodulatory effects, this suggests that the antitumor activity seen in these animal models may be mediated by the inhibition of FAP at the tumor microenvironment.

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